RETROVIRAL-MEDIATED TRANSFER

OF SUPPRESSOR tRNA GENES INTO HUMAN CELLS

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by

Barbara Janine Breithaupt

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Nonsense mutations in protein genes have been associated with a variety of human diseases. A useful therapeutic approach to correct such mutations may be to introduce genes expressing suppressor tRNA that recognize the premature stop codon, insert the appropriate amino acid, and allow translation of a full-length, functional protein. Human opal arginine suppressor tRNA genes that encode the structural portion of the tRNA and about 15 bases from the 3' flanking sequence were synthetically constructed using oligonucleotides. Retroviral vectors were selected for delivery to human target cells in order to stably integrate the suppressor tRNA into the target cells' genome. Recombinant retroviral vectors were used to transfect retrovirus packaging cells and produce recombinant viral supernatants. Recombinant viral supernatants were then used to transduce the target cells. Expression of suppressor tRNA in both the retrovirus packaging cells and in the target cells was confirmed by slot blot analysis. To test the functional activity of the expressed suppressor tRNA, target cells expressing a nonsense mutated GFP reporter gene (Arg73opal mhRGFP) were transduced with the recombinant viral supernatant. Suppression of the opal nonsense mutation in hRGFP was demonstrated by restoration of hRGFP fluorescence. This study suggests that retroviral vectors may be used to deliver suppressor tRNA genes to human target cells.

A Part of Nature

Sometimes we see a part of Nature
meant for eyes of God alone,
and as long as mercy meek shall be,
the moment is eternity,
for as an eagle rises hungry in the blue
o'er winter's oldest snows,
so my thoughts from depths of day
shall live again that view.

Edward Eylar, Ph.D.
from a collection of poems, REALITY AND DNA
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ABBREVIATIONS

3'	3'-OH group of d-ribose
5'	5'-carbon of d-ribose
A	adenine
amber	UAG nonsense codon (terminator codon) found in mRNA
amp	ampicillin resistance gene (beta-lactamase)
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cfu	colony-forming unit
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
D-MEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP
DTT	dithiothreitol
E. coli	*Escherichia coli*
env	viral gene for envelope protein
EDTA	ethylene diaminetetraacetic acid
FBS	fetal bovine serum

gag	retroviral gene for glucosaminoglycan core protein
G	guanine
G418	Geneticin, an aminoglycoside related to gentamicin sulfate
GFP	green fluorescent protein (*Aequorea victoria*)
GP+E-86	retroviral packaging cell line with ecotropic host range
hr	hour
HBSS	Hank's Balanced Salt Solution
hRGFP	humanized red-shifted green fluorescent protein
int	retroviral gene region encoding a protein involved in the integration of viral DNA into the host genome
kb	kilo base pairs
LB	Luria broth
LTR	viral long terminal repeat
M	Molar
mdx mice	animal model for human Duchenne muscular dystrophy
mhRGFP	mutated humanized red-shifted green fluorescent protein
min	minute(s)
ml	milliliter
mM	millimolar
mmol	millimole
MOI	multiplicity of infection
mol	mole

MoMuLV	Moloney murine leukemia virus
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NIH/3T3	contact-inhibited cells established from NIH Swiss mouse embryo cultures, ATTC CRL-1658
nm	nanometer
nmol	nanomole
neo	neomycin phosphotransferase gene
ocher	UAA nonsense codon (terminator codon) found in mRNA
opal	UGA nonsense codon (terminator codon) found in mRNA
PA317	retroviral packaging cell line with amphotropic host range
pmol	picomole
pol	retroviral gene region encoding reverse transcriptase and a protein needed for viral DNA integration into the host genome
pro	retroviral gene region encoding a protease needed for cleavage of the *gag* and *pol* polyproteins
Psi+	extended viral packaging signal
Ψ+	extended viral packaging signal
RGP34	oligonucleotide forming the 5' end of opal arg tRNA gene
RGP49	oligonucleotide forming the 3' end of opal arg tRNA gene
RNA	ribonucleic acid
RT	room temperature

sec	second(s)
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
SV40	simian virus 40
T	thymine
T7	bacteriophage T7
TAE	Tris-acetate, EDTA buffer
TE	Tris, EDTA buffer
TFIII	group of transcription factors for eukaryotic RNA polymerase III promoters
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	uracil
μg	microgram
μl	microliter
μM	micromolar
μmol	micromole

INTRODUCTION

Gene therapy describes the treatment of human disease by transferring genetic material into a patient. The genetic material may be either DNA or RNA intended for somatic or germ line therapy (Polvino and Anderson, 1996). Gene therapy directed towards the production of functional proteins could follow either of two approaches. The treatment may introduce a normal gene or a gene with a therapeutic effect.

In replacement therapy, a functional gene is introduced in the organism to complement the defective gene, thereby restoring the normal phenotype (Anderson, 1992). This approach has been tested *in vitro* and *in vivo* for a range of diseases such as adenosine deaminase deficiency (Blaese et al., 1995), Xeroderma pigmentosum (Panchal et al., 1999), Cystic fibrosis (Yagi et al., 1998), and Type 1 Diabetes (Gros et al., 1999).

Genes with potential therapeutic effects are also being studied for use in the treatment of diseases. For example, suicide genes encoding prodrug-activating enzymes such as herpes simplex thymidine kinase in combination with the prodrug ganciclovir, are being studied for the treatment of colon adenocarcinoma (Nielsen et al., 1997). Other strategies include the use of antisense oligonuclotides, ribozymes, and cytokine gene therapy (Lattime and Gerson, 1999).

The cloning and characterization of genes has revealed a wide spectrum of diseases that occur as a result of nonsense mutation (Atkinson and Martin, 1994). The international effort to map the human provides information for normal genotypes that can be utilized for development of therapeutic strategies for the disease phenotypes (Jonsen,

1996). Proteins are normally translated from messenger RNA (mRNA) which have sequences coding for the structural portions of the protein followed by one of three translation termination signals: amber (UAG), ocher (UAA), or opal (UGA) stop codons. Mutations in the portion of the mRNA coding for the structural part of the protein which produce a premature termination signal are termed nonsense mutations. As expected, this type of mutation will ultimately cause premature termination of protein synthesis. The resulting truncated protein may have limited function or may not have any normal function. Gene therapy may play a role in the treatment of diseases resulting from nonsense mutations through the use of suppressor tRNA genes that are designed to recognize the nonsense codon, insert the amino acid which would be present in the normal phenotype, and continue protein translation beyond the nonsense mutation.

The potential application of suppressor tRNA for gene therapy was first demonstrated for β-thalassaemia. Temple et al. (1982), coinjected the nuclei of *Xenopus laevis* oocytes with amber lysine suppressor tRNA and mRNA from a patient with β-thalassaemia. The suppressor tRNA was able to suppress the nonsense mutation in the β-globin gene and synthesize a full-length β-globin gene product. Another strategy used suppressor tRNA to control the expression of the nonsense-mutated diphtheria toxin A gene in mammalian cells and thereby target ablation of cancerous tissues (Robinson and Maxwell, 1995). The *in vivo* application of suppressor tRNA for treatment of genetic disease caused by nonsense mutation was demonstrated in an mdx mouse, an animal model for human Duchenne muscular dystrophy with an ochre mutation in the dystrophin

gene. Direct injection of plasmid DNA, encoding the ochre suppressor tRNA, into mdx mouse tissue produced dystrophin positive fibers (Li et al., 1997).

A major challenge to the success of gene therapy is the development of safe and efficient delivery systems for the transfer of therapeutic gene(s) into target cells. To achieve this goal, various recombinant viral and non-viral delivery systems have been developed (Robbins and Ghivizzani, 1998).

The aim of this study is to determine if recombinant retroviral vectors can be used as delivery vehicles to transfer opal arginine suppressor tRNA genes into human cells using accepted methods for replication-incompetent retroviral vectors and packaging cell lines.

Project Overview

This investigation began with the synthesis of opal arginine suppressor tRNA genes. The synthetic gene inserts were subcloned into a replication-incompetent retroviral vector, pLNXpL1. The recombinant vectors bearing plausible suppressor tRNA genes were identified by endonuclease restriction analysis. After the initial screening, the vector constructs were tested for function using a nonsense-mutated reporter gene, humanized red-shifted green fluorescent protein (mhRGFP). Plasmid constructs bearing functional suppressor tRNA genes were verified by DNA sequencing. Retroviral vectors with functional suppressor tRNA genes were transfected into an ecotropic packaging cell line, GP+E-86, to produce viral particles (virions) capable of infecting mouse cells. Virions produced by GP+E-86 were then used to transduce an amphotropic packaging cell line, PA317, to produce virions capable of transferring the therapeutic gene to human cells. A

viral titer bioassay was used to monitor the production of virions by PA317. Supernatant containing virions was used to transduce human target cells in an attempt to stably integrate the suppressor tRNA gene in the host cells' genome. Expression of opal arg suppressor tRNA genes in both the packaging cells and in human target cells was evaluated by slot blot analysis. XP12ROSV human target cells were transfected with the mutated hRGFP gene and then transduced with the viral supernatant produced by PA317 cells. Restoration of GFP fluorescence in the target cells would demonstrate suppression of opal nonsense mutation and translation of a full-length, functional hRGFP protein.

REVIEW OF THE LITERATURE

tRNA

Transfer RNA (tRNA) are a class of molecules that mediate the insertion of amino acids into the nascent polypeptide chain by sequential identification of codons on mRNA. Every cell contains a set of tRNAs, each of which carry one of the 20 different amino acids to the site of protein translation.

Mature tRNAs are a small RNA molecules, usually 70 to 90 nucleotides in length. Nucleotides within each molecule have extensive hydrogen bonding to form a cloverleaf secondary structure of four helical segments and three loops (Figure 1). The molecule is further folded into an L-shape. tRNAs are transcribed by RNA polymerase III and contain their own intragenic split promoters that become a part of the mature tRNA coding sequence (Sharp et al, 1985; Geiduschek and Tocchini-Valentini, 1988). Two structural features of tRNA that are important in protein translation are the 3' amino acid acceptor stem and the trinucleotide anticodon loop. The 3' acceptor stem of the tRNA consists of four nucleotides in a single strand ending with CCA_{OH}. An aminoacyl-tRNA synthetase identifies recognition elements on the structural portion of the tRNA and attaches a specific amino acid by ester linkage to the 3' adenosine of the CCA sequence (Saks et al., 1994). The anticodon loop is complementary to the amino acid codon on mRNA with some flexibility in base pairing (Schimmel and Ribas de Pouplana, 1995). Some amino acids have more than one tRNA in order to accommodate the different mRNA codons for the same amino acid.

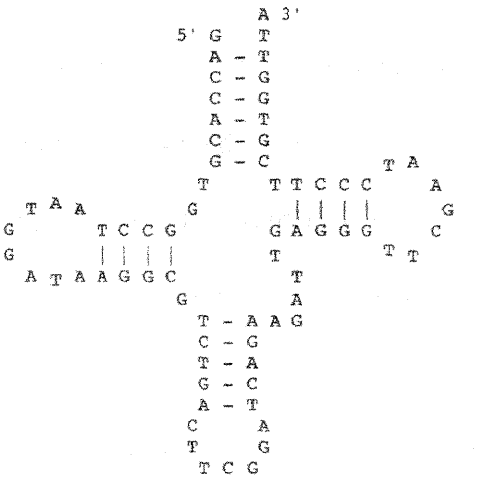

Figure 1. The noncoding strand of the human arginine tRNA gene shown in cloverleaf formation (adapted from Buckland et al., 1996).

Natural human tRNA genes are considerably larger than the mature structural form. The 5' and 3' flanking sequences appear to provide extragenic transcriptional control. Arnold and Gross (1987) described a positive control element between the -51 and -16 nucleotide positions which enhanced the formation of stable transcription complexes. In another study, Arnold et al., 1988, subsequently observed that the 3' extragenic control element appeared to stimulate transcription of the tRNA by facilitating the transition of a preinitiation complex into an active transcription complex.

The human arginine tRNA has been sequenced and mapped to the short arm of chromosome 6 using recombinant libraries (Buckland et al., 1996). There appears to be a cluster of low-copy tRNA genes, including methionine, alanine and arginine tRNA genes, in the 6p21.2-p22.3 region. The opal arg suppressor tRNA used in this study has a design based on the structural sequence of the human arginine tRNA gene.

Suppressor tRNA

Suppressor tRNAs are a form of tRNA that base pair with a termination codon in mRNA and add an amino acid to what would have been the C terminus (carboxy terminus) of the nascent polypeptide chain. Translation of the protein is not terminated at the stop codon, but instead, proceeds to one of the alternate stop codons on the mRNA. The suppressor tRNA are named on the basis of which type of stop codon is paired to the anticodon during translation: UAG (amber), UAA (ochre), or UGA (opal).

Suppressor tRNAs are known to occur naturally in both prokaryotes and some eukaryotes. There has been extensive characterization of suppressor tRNA in bacteria, yeast, and ciliate protozoa. In *Saccharomyces cerevisiae,* glutamine suppressor tRNA are found to suppress amber and ocher stop codons (Weiss et al., 1987). Early studies (Kuchino et al., 1985; Hanyu et al., 1986) suggest that glutamine tRNA may be an evolutionary progenitor of amber suppressor tRNA. Amber or ocher glutamine suppressor tRNA are found in *Tetrahymena thermophila.* Their findings indicate that there are three types of glutamine tRNA: two recognize the normal CAA and CAG codons for glutamine, the third recognizes both UAA and UAG stop codons.

Mammalian suppressor tRNAs have been described. Valle et al. (1987) isolated amber leucine tRNA from calf liver. Kuchino and Muramatsu (1996) isolated amber glutamine suppressor tRNA from mouse and rat livers. They described conditional suppression when unique pseudoknot structures, required for nonsense suppression, were present in mRNA.

The human genome has been screened for naturally occurring suppressor tRNA. O'Neil (1985) described an opal serine suppressor tRNA from a human DNA library which was detected by an opal suppressor tRNA probe. Opal serine suppressor tRNAs have been found in mouse, bovine, and human tissues (Hatfield et al., 1990)

To date, a review of the literature does not reveal any naturally occurring human opal arginine suppressor tRNA. In addition, a database search of the Online Mendelian Inheritance in Man revealed more than 200 reports of an arginine to opal mutation in genes, thereby leading to disease conditions. This finding appears to support the probability that opal arginine suppressor tRNAs are not naturally found in the human genome. In addition, the cataloging and characterization of the genetic basis for these diseases reaffirms the need for therapeutic intervention.

Functional suppressor tRNA could be either the full-length gene of about 800 nucleotides that include the 5' and 3' flanking sequences or only the mature form of about 100 nucleotides. Different suppressor tRNAs could be constructed using oligonucleotide site-specific mutagenesis to change one or two nucleotides in the anticodon of the cloned tRNA gene (Capone et al., 1985). Amber phenylalanine and amber cysteine suppressor genes were synthetically constructed using four to six oligonucleotides (Normanly et al., 1986). These genes did not contain the 5' and 3' flanking sequences, however, they were able to suppress an amber mutation in the dihydrofolate reductase protein gene in *E. coli*. Both the full-length and the mature form of the suppressor tRNA genes would be within the size limitations of a retroviral vector system.

Retroviral vectors

The production of a functional protein from a gene with a nonsense mutation would benefit from long-term expression of a therapeutic suppressor tRNA. For this study, recombinant retroviral vectors were selected as delivery vehicles because they have been shown to stably integrate the therapeutic gene into human somatic cells (Morgenstern and Land, 1991).

The retroviral vector used in this study is based on the Moloney murine leukemia virus (MoMuLV). In general, the MoMuLV life cycle begins with virion attachment to a specific cell-surface receptor, followed by fusion of the envelope protein and endocytosis into the host cell. The RNA viral genome is reverse transcribed to a linear duplex of DNA within the virion core and transferred into the host cell's nucleus while still associated with virion proteins. Viral DNA is integrated at random sites in the host's genome to form the provirus. Replication-competent viruses have genes which code for the viral proteins: *gag*, *pro*, *pol*, and *env* which are required for the synthesis of viral particles. The *int* gene product of the *pol* region mediates the integration of proviral DNA into the genome of the host. The viral genome is then synthesized from the 5' LTR region to the 3' LTR region. The 5' LTR is recognized by the host's cellular transcription machinery and acts as a promoter for the viral genome using the host's RNA polymerase II. The full-length RNA transcripts are fated for the new viral genome or are processed to mRNAs for *gag*, *pro*, and *pol*, and *env* viral proteins. Finally, virion proteins are synthesized, and along with the RNA genome, assembled into capsids. Replication-competent viruses produce infectious

particles which bud from the host cell and undergo further rounds of host cell infection (Miller, 1990; Figure 2).

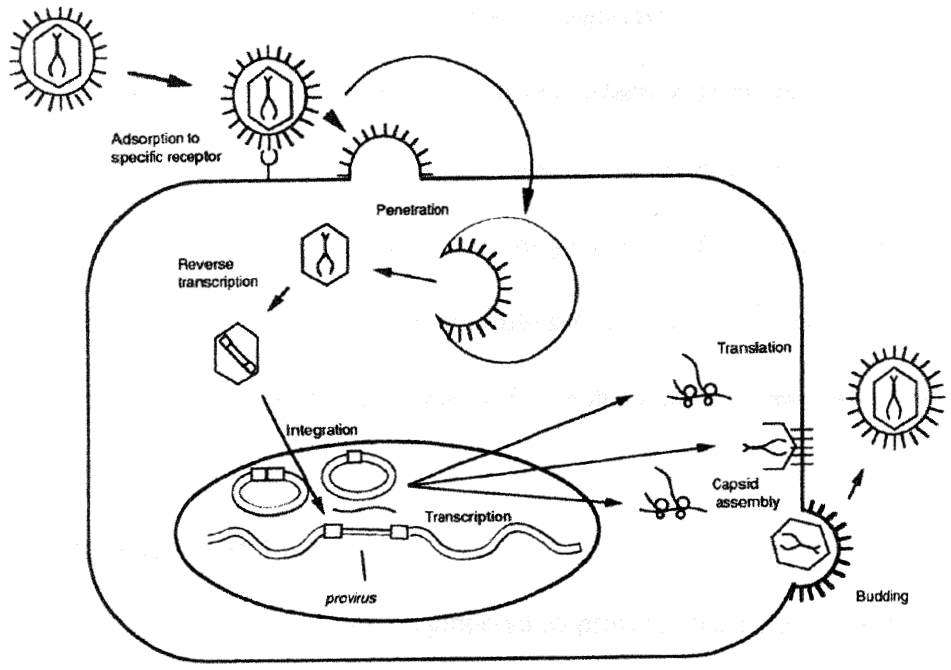

Figure 2. Replication cycle of retroviruses (adapted from Fields et al., 1996)

Retroviral vectors have been engineered for safety, to be replication-incompetent. The use of replication-competent viruses could result in multiple random integration of viral DNA into the host genome. The random integration could disrupt normal gene expression or activate oncogenes (Varmus et al., 1981). Moloney murine leukemia virus has been recombined to generate a replication-incompetent retroviral vector by separating from the retrovirus, partially or completely, *cis*-acting viral genes needed for the production of infectious particles. The remaining 5' LTR, Ψ^+ packaging signal, and 3' LTR proviral DNA are recombined into a plasmid backbone. The structural proteins that would have been provided by the retroviruses' own genes are supplied in *trans* by

"packaging cells" to generate virions. The viral regulatory sequence present in the 5' LTR, or an independent promoter, for example the CMV or SV40 promoter, subcloned into the retroviral vector may be used to express a therapeutic gene of interest. In addition, the viral vector may also contain antibiotic selection markers which allow for identification of infected clones or enable the generation of populations of cells which consist entirely of transduced cells (Eglitis and Anderson, 1988). For example, an ampicillin resistance gene is often used for selection in prokaryotic cells, while the neomycin resistance gene confers resistance to G418 in eukaryotic cells (Miller et al., 1988).

Retroviral packaging cell lines

Packaging cell lines have been engineered to provide structural proteins needed to generate virions for replication-incompetent retroviral vectors. Complementary *gag-pol* and *env* coding regions were incorporated into NIH/3T3 mouse fibroblast cells to generate packaging cells capable of producing infectious particles by supplying the viral proteins needed for the production of infectious particles which had been partially or totally removed from the retrovirus. The resulting GP+E-86 ecotropic host range cell line and PA317 amphotropic host range cell line provide the viral structural proteins in *trans* so that infectious particles can be produced only within the packaging cell line (Miller and Buttimore, 1986; Danos and Mulligan, 1988).

Two packaging cell lines have been employed in this study in order to minimize the potential for the development of replication-competent retroviral particles and maximize the potential for production of high-titer vector producing cells (Markowitz et al., 1988).

GP+E-86, which initially receives the recombinant plasmid, was designed to separate the *gag-pol* genes from the *env* gene as an additional barrier to the formation of

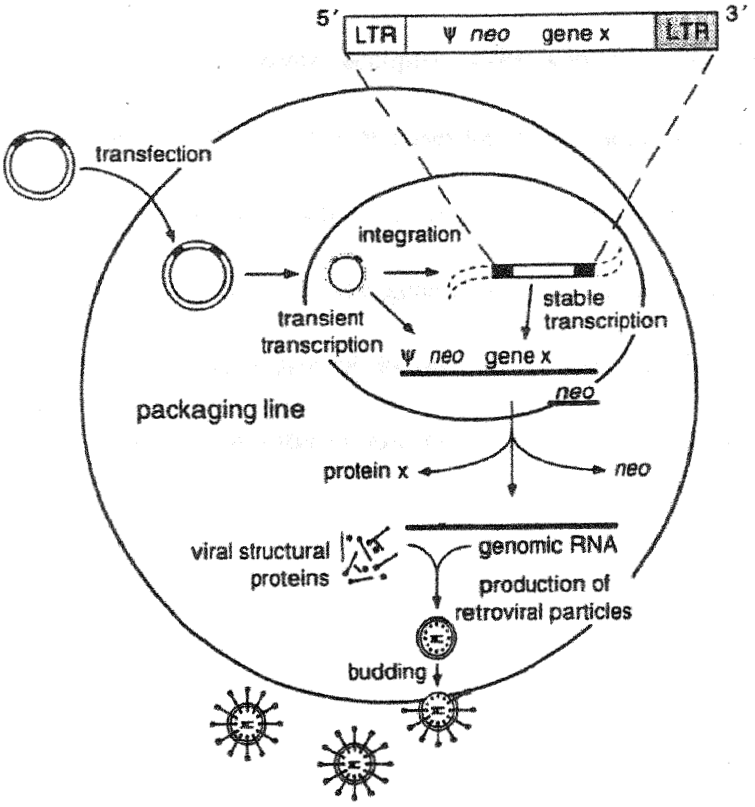

Figure 3. Recombinant retroviral plasmid transfected into a packaging cell line resulting in the production of virions (adapted from Ausubel et al., 1996)

replication-competent retroviruses (Figure 3). Because this packaging cell line has an ecotropic host range, virions produced by GP+E-86 are not capable of infecting human cells. In order to produce virions capable of infecting human cells, viral supernatant produced by GP+E-86 cells is used to transduce PA317 cells. The production of virions using two packaging cell lines will increase the production of virions and also benefit from the added safety barrier to replication-competent retroviral infection.

hRGFP as a Reporter Gene

Green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* has been widely used as a reporter protein to investigate gene transfer. GFP is a 238 amino acid polypeptide that acts as an energy-transfer acceptor. GFP is excited with blue light at 488 nm and produces emissions at 525 nm. Retroviruses have been successfully used to transfer GFP into human cells and allow detection of cells expressing the transgene to be identified (Levy et al., 1996). Modifications in the gene coding for the protein have resulted in the human codon optimized version of the protein. Additional changes have generated a variety of GFP proteins that differ in their excitation and emission spectra.

MATERIALS AND METHODS

Reagents

All reagents described are given at final concentration.

Cell lines

GP+E-86. (ATCC, CRL-9642) Retroviral packaging cells derived from NIH/3T3 with an ecotropic range of *env* genes, a class of viral glycoproteins that permit virions to bind the receptor found only on rat and mouse cells (Markowitz et al., 1988).

PA317. (ATCC, CRL-9078) Retroviral packaging cells derived from NIH/3T3 with an amphotropic class of *env* genes with a broad host range including rodents, mice, chickens, dogs, cats, and humans (Miller and Buttimore, 1986).

XP12ROSV. SV40-transformed cell lines established from a patient with Xeroderma pigmentosa (a kind gift from K. Tanaka, Japan).

IGROV. Human ovarian carcinoma cells (a kind gift from P. Hwe, National Cancer Institute, USA).

Tissue culture

All tissue culture was performed in a biological safety cabinet Model 1104 (Forma Scientific) using sterile gloves and technique. Tissue culture was performed in 6-well plates (well diameter 35mm, Corning) or T-80 tissue culture flasks (Nunc). Volumes will be given in the format: 35mm well/T-80 flask. All cell lines were grown in Dulbecco's Modified Eagle Medium (Gibco BRL) medium, supplemented with heat-inactivated fetal bovine serum (10%, Gibco BRL), penicillin (100 units/ml, Gibco BRL), streptomycin (100

μg/ml, Gibco BRL), and L-glutamine (292 μg/ml, Gibco BRL) (complete D-MEM). Cells were incubated at 37°C, 80% humidity, 5% CO_2 in a STERI-CULT 200 incubator (Forma Scientific). Cells were passaged when confluent by aspirating the medium, washing once with 2 ml/10 ml Hanks' Balanced Salt Solutions (HBSS, Gibco BRL), followed by incubation of cells in 0.5 ml/2.5 ml 0.05% trypsin, 0.53 mM EDTA•4 Na (Gibco BRL) until the cells detached when gently agitated. Complete D-MEM, 1 ml/5 ml, was added to the culture. Viable cells were counted using an appropriate dilution with trypan blue (Sigma Chemical Co.) and a hemacytometer. Cells were reseeded into 2 ml/15 ml of complete D-MEM or as specified in a procedure (Martin, 1994).

Gene transfer techniques

Transformation. Transformations were used to isolate plasmid clones or to generate a working stock of recombinant plasmids. DH10B *E. coli* competent cells (Gibco BRL) were transformed with recombinant plasmid following the manufacturer's recommended transformation procedure. DH10B cells were thawed over ice. The recombinant vector ligation mixture (10 μl) or plasmid DNA (~2 μl) and DH10B cells (100 μl) were incubated on ice for 40 minutes, then heat-shocked at 42°C for one minute. The transformed DH10B cells were spread on LB-ampicillin (100μg/ml) agar plates, and incubated overnight at 37°C in a bacterial incubator.

Transfections. Transfection was carried out using FuGENE 6 reagent (Boehringer Mannheim) following the manufacturer's guidelines. Mammalian cells (2×10^5) were seeded into each 35 mm well of 6-well tissue culture plate (Corning) and allowed to attach

for 16 to 20 hr. The medium was aspirated and replaced with 2 ml fresh complete D-MEM. FuGENE 6 transfection reagent (12 µl) was first incubated with Opti-MEM (88 µl, Gibco BRL) for 5 minutes at RT, then added to 4 µg plasmid DNA diluted with TE buffer (10mM Tris/HCl, 1mM EDTA, pH 8.0) to a volume of 10 µl and further incubated for 15 minutes at RT. The transfection mixture was added to the cells cultured in the 35 mm well. After 14 to 16 hr at 37°C, the cells were washed once with 2 ml HBSS, then fed with 2 ml complete D-MEM and further incubated for 24 to 48 hr.

Transduction. Medium was first removed from the target mammalian cells cultured in 35 mm wells of 6-well plates (Corning). The supernatant from transfected GP+E-86 or PA317 packaging cells was collected in a sterile syringe, 1 ml was passed through a 0.22 µm µSTAR LB filter (COSTAR) and added to the target mammalian cells. Protamine sulfate (10 µg) was added to each ml of supernatant. Fresh complete D-MEM, 1 ml, was replaced over the GP+E-86 or PA317 packaging cells. Successive transductions were carried out at twelve-hour intervals for a total of six transductions in three days. Twelve hours after the final transduction, the medium over the target cells was removed, the cells were washed with 2 ml HBSS, and the medium replaced with 2 ml complete D-MEM or complete D-MEM plus G418 (Geneticin, 1mg/ml, Gibco BRL) to begin selection of cells which contained the recombinant plasmid.

Synthesis of Opal Arginine Suppressor tRNA Gene Insert

The opal arginine suppressor tRNA gene was designed based on the human arginine tRNA structural sequence (Buckland et al., 1996). A single base change in the anticodon altered the human arginine tRNA to function as an opal suppressor tRNA. A human opal arginine suppressor tRNA gene encoding the structural portion of the molecule and 15 bases of the 3' flanking region was synthesized using a pair of overlapping oligonucleotides. In addition, the suppressor tRNA design also included nucleotide sequences that would generate endonuclease restriction sites at the termini to enable subcloning into the retroviral vector.

The opal arginine suppressor tRNA genes used for this study were constructed from two commercially synthesized oligonucleotides (Integrated DNA Technologies, Inc.). The 5' end of the suppressor tRNA gene was formed by oligonucleotide RGP34: 5' gcgctcgagaaaacgaaccccacttaaccacgaagggattcgaaccctcaatcttctgatc 3'. The 3' end of the suppressor tRNA gene was formed by oligonucleotide RGP49: 5' gcgaagcttgaccacgtggcctaatggataaggcgtctgacttcagatcagaagattgaggg 3'.

The two oligonucleotides were annealed at their complementary regions by combining 1 nmol RGP34 and 1 nmol RGP49 in a reaction mixture containing 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl, for a final volume 20 µl. The reaction mixture was heated to 65°C for two min in a heat block. Oligonucleotides were annealed by slow cooling to 40°C over 30 min, then placing the reaction mixture on ice.

The single-stranded DNA bases on the annealed oligonucleotides were filled-in using a reaction mixture containing T7 DNA polymerase enzyme (Sequenase Version 2,

United States Biologicals) and all four deoxynucleotides. DTT (7.7mM, Gibco BRL); dNTP (dATP, dCTP, dGTP, and dTTP, each at a final concentration of 0.77 mM; Gibco BRL), and 3.25 units of T7 Sequenase enzyme, diluted according to the manufacturer's protocol in 1.7 µl of 50 mM Tris-HCl pH 7.5, and 10 mM 2-mercaptoethanol, final volume 26 µl. This reaction was incubated at RT for six min. The enzyme was then heat-inactivated at 75°C for three min. This resulted in the double-stranded DNA for the opal arginine suppressor tRNA gene insert subcloned into the pLNXpL1 plasmid (Figure 4).

```
5' gcgctcgagaaaacgaaccccacttaaccacgaagggattcgaaccctcaatcttctgatctgaagtcaga
   cgcgagctctttgcttggggtgaattggtgcttccctaagcttgggacttagaagactagacttcagtct
      Xho I
   cgccttatccattaggccacgtggtcaagcttcgc 3'
   gcggaataggtaatccggtgcaccagttcgaagcg
                              Hind III
```

Figure 4. Double-stranded DNA for the opal arginine suppressor tRNA gene subcloned into the pLNXpL1 plasmid.

The ends of the double-stranded DNA were prepared for subcloning into the retroviral vector by digestion with restriction endonucleases *Xho* I and *Hin*d III. 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 25 units of *Hin*d III (Gibco BRL), and 25 units of *Xho* I (Gibco BRL) were added to the double-stranded DNA synthesized in the fill-in reaction, final volume 50 µl. The reaction mixture was incubated in a waterbath at 37°C for two hr. The digested opal arginine tRNA gene insert was transferred to a Chroma Spin+TE-30 column (Clontech) to remove unincorporated nucleotides and small oligonucleotide fragments using the manufacturer's protocol.

The DNA was ethanol precipitated as described by Sambrook et al., 1989. In brief, the volume of DNA in buffer recovered from the Chroma Spin+TE-30 column was measured. Two volumes of ice-cold 100% ethanol, 0.1 volume of 4M ammonium acetate, and 0.01 volume of 20 mg/ml glycogen was added to the DNA recovered from the Chroma Spin column. This mixture was placed into the -70°C freezer for 15 min to precipitate the DNA. The mixture was centrifuged at 12,000 X g for 15 minutes and the supernatant poured off. 200 µl of ice-cold 70% ethanol was carefully added so as not to disturb the pellet, and immediately poured off. 200 µl of ice-cold 95% ethanol was added and immediately poured off. The pellet was allowed to air dry briefly, then resuspended in 100 µl TE buffer. 1 µl of resuspended DNA was added to 99 µl of TE, pipetted into a 100 µl cuvette and quantitated by spectrophotometer (Beckman DU Series 500) reading at OD_{260} using the manufacturer's protocol.

The pLNCX retroviral vector (Clontech) was modified to remove the CMV promoter and introduce a polylinker containing multiple cloning sites (kind gift of Robert Mandell). The resulting pLNXpL1 vector was used in this study. Because eukaryotic tRNAs are known to have a split intragenic promoter (Sharp et al., 1985), the pLNXpL1 retroviral vector should demonstrate tRNA gene expression without an extragenic promoter.

pLNXpL1 plasmid was digested with *Xho* I and *Hin*d III to open the plasmid and generate ligation sites compatible with the opal arginine tRNA gene insert. pLNXpL1 plasmid DNA (5 pmol), 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, and 25

units of each *Xho* I and *Hind* III, final volume 50 µl, were incubated in a water bath at 37°C for two hr. The digested plasmid DNA was electrophoresed over 1% low melting point (65°C) agarose (Sigma) in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). The band corresponding to 5851 bp was cut from the gel and quantitatively transferred to a SPIN-X 0.22 µm cellulose acetate centrifuge filter (COSTAR) using 200 µl TE buffer. After centrifugation at 10,000 X g for three minutes, the DNA was ethanol precipitated as described previously, resuspended in 20 µl TE buffer. The quantity of DNA was estimated by comparing the relative band density of 1 µl 1 Kb DNA ladder (1 µg/µl, Gibco BRL) to a 2 µl plasmid DNA sample after both had been electrophoresed on a 1% low melting point agarose gel prepared with 1X TAE.

The gene insert was ligated into the pLNXpL1 vector using T4 DNA ligase enzyme and reagents accompanying the enzyme (New England BioLabs, Inc.). The reaction mixture containing 100 ng digested pLNXpL1 plasmid vector, a five-fold molar excess of opal arginine tRNA gene insert, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 25 mg/ml BSA, and 400 units T4 DNA ligase enzyme, reaction pH of 7.8, final volume 10 µl. This mixture was ligated overnight in a 14°C water bath. The ligation mixture was then used to transform competent *E. coli* as previously described in this manuscript.

Screening of bacterial colonies for plasmids containing suppressor tRNA

Recombinant plasmids were screened by using restriction enzymes to digest the plasmid fragment which contained the multiple cloning site and suppressor tRNA gene

insert. To screen for plasmids containing suppressor tRNA, individual bacterial colonies were picked from the LB-ampicillin plates, innoculated into 5 ml aliquots of LB-ampicillin medium (250 μg/ml), and incubated overnight at 37°C in a bacterial shaker. Plasmid DNA was isolated using the Wizard Plus miniprep purification kit (Promega) following the manufacturer's protocol. Each DNA miniprep was digested with 10 units *Kpn* I (Gibco BRL), 10 units *Xho* I (Gibco BRL), 35 mM Tris-HCl pH 7.7, 7.5 mM $MgCl_2$, 25 mM NaCl, and 25 mM KCl, final volume 20 μl. The reaction was incubated for two hr at 37°C in a water bath. Digested DNA samples were electrophoresed on a 1% low melting point agarose gel in 1X TAE at 100V for 45 min. The gel was stained with ethidium bromide by immersing the electrophoresis gel into a solution of 50 μg ethidium bromide in 500 ml TAE buffer, for 15 min. The gel was removed from the staining solution, placed on a 365 nm UV light box (Fotodyne) and photographed. The electrophoretic banding patterns were inspected to determine the presence of bands corresponding to a base-pair size equivalent of the region of the plasmid multiple cloning site and opal arg suppressor tRNA gene insert. Plasmid clones which contained the suppressor tRNA gene insert would have restriction digest fragments approximately 100 bp larger than fragments from plasmid clones without the gene insert. Those DNA plasmid clones bearing plausable suppressor tRNA gene inserts were selected for assessment of the functional activity of the suppressor tRNA.

Assay for functional activity of suppressor tRNA

Contruction of mhRGFP reporter gene . An opal mutation was introduced in the humanized red-shifted green fluorescent protein (hRGFP) gene by polymerase chain reaction. The arginine codon at amino acid position 73 in the hRGFP gene was changed to an opal stop codon (UGA) and subcloned into a pHE700 vector (pHE700 Arg73opal mhRGFP, kind gift of Rekha Panchal).

In order to subclone the mhRGFP gene into a pLNCX retroviral vector, the gene was excised from the pHE700 vector by a two-step digestion. In the first step, the pHE700 vector was digested with the *Not* I enzyme. A reaction mixture containing 10 µg pHE700arg73opal hRGFP plasmid DNA, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, and 30 units of *Not* I, final volume 20 µl, was incubated 2 hr at 37°C.

The overhanging nucleotides were filled-in using a reaction mixture containing T7 DNA polymerase enzyme (Sequenase Version 2, United States Biologicals) and all four deoxynucleotides. The reaction mixture contained DTT (7.7mM, Gibco BRL); dNTP (dATP, dCTP, dGTP, and dTTP, each at a final concentration of 0.77 mM; Gibco BRL), and 3.25 units of T7 Sequenase enzyme, diluted according to the manufacturer's protocol in 1.7 µl of 50 mM Tris-HCl pH 7.5, and 10 mM 2-mercaptoethanol, final volume 26 µl.

The linearized plasmid was subsequently digested in a mixture containing 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, and 20 units of *Hind* III, final volume 50 µl, and incubated two hr at 37°C. The digested fragments were gel purified by electrophoresis on a 1% low melting point agarose gel prepared with 1X TAE to separate the mhRGFP gene insert from other digestion fragments. Only one 737 bp band,

corresponding to the size of the mhRGFP gene, was cut from the gel and purified using a SPIN-X 0.22 mm cellulose acetate centrifuge filter (COSTAR). The mhRGFP gene DNA fragment was ethanol precipitated as previously described, and resuspended in 20µl TE.

Retroviral pLNCX plasmid (Clontech) was prepared for subcloning the mhRGFP gene by first digesting it with 20 units of *Cla* I in a reaction mixture containing 50 mM Tris-HCl pH 8.0, and 10 mM $MgCl_2$, final volume 20 µl, and incubated at 37°C for two hr. The overhanging nucleotides were filled-in as previously described. The linearized, blunt-ended pLNCX vector was gel purified, and ethanol precipitated. The cut ends of the linearized plasmid were prepared for ligation by digestion in a mixture containing 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 nM NaCl, and 20 units of *Hin*d III, final volume 50 µl, and incubated two hr at 37°C.

The digested mhRGFP fragment was ligated to the linearized pLNCX (Clontech) using T4 DNA ligase and reagents accompanying the enzyme (New England BioLabs, Inc.) using overhanging end of the *Hin*d III site and the blunt end generated by the fill-in of the *Cla* I restriction site. The reaction mixture containing 100 ng digested pLNCX plasmid vector, a five-fold molar excess of mhRGFP gene insert, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 25 mg/ml BSA, and 400 units T4 DNA ligase enzyme, reaction pH of 7.8, final volume 10 µl, was ligated overnight in a 14°C water bath. The recombinant plasmid, pLNCX mhRGFP, was used to test the functional activity of opal arg tRNA (Figure 5).

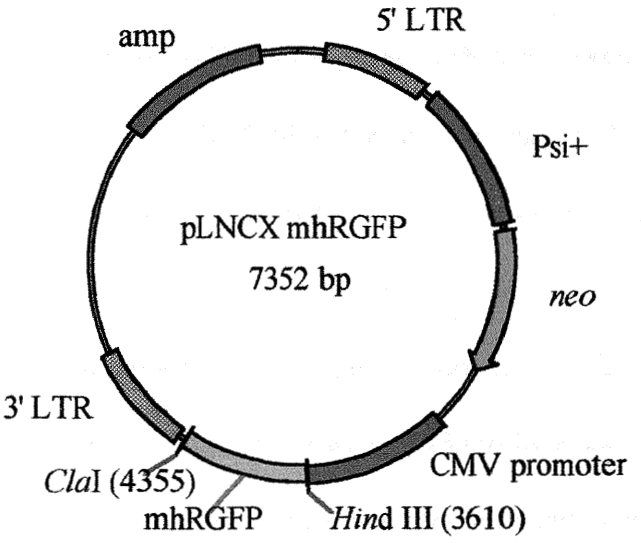

Figure 5. The linearized arg73opal mhRGFP reporter gene was subcloned into the retroviral vector pLNCX to generate the recombinant plasmid pLNCX mhRGFP used to establish functional activity of opal arginine suppressor tRNA.

Testing for functional activity of suppressor tRNA. Plasmid DNA with a plausible opal arginine suppressor tRNA gene insert and pLNCX arg73opal mhRGFP were cotransfected into XP12ROSV cells. A combination of suppressor tRNA and mhRGFP DNA was used for the cotransfection. Cells cotransfected with functional gene inserts would be expected to exhibit green fluorescence.

XP12ROSV cells were seeded into 6-well culture plates (Falcon) at 2 X 10^5 cells per 35 mm well, a density sufficient to generate a 50% confluent surface in 24 hr. Each cotransfection reaction consisted of 10 µl suppressor tRNA miniprep plasmid DNA, 2 µg pLNCX arg73opal mhRGFP, 6 µl FuGENE 6 transfection reagent (Boehringer Mannheim), and 94 µl OptiMEM (Gibco BRL). At 24 and 48 hours, the medium was removed from the wells of the culture plate and replaced with 2 ml HBSS. The cotransfected cells were observed at 100 X magnification using a Diaphot 300 fluorescent microscope (Nikon) illuminated with a 488 nm light source. GFP-positive cells, visible as bright green cells, indicated which plasmid clones contained functional opal arg tRNA. The recombinant retroviral vector containing functional opal arginine suppressor tRNA was designated pLNXpL1 OAT (Figure 6).

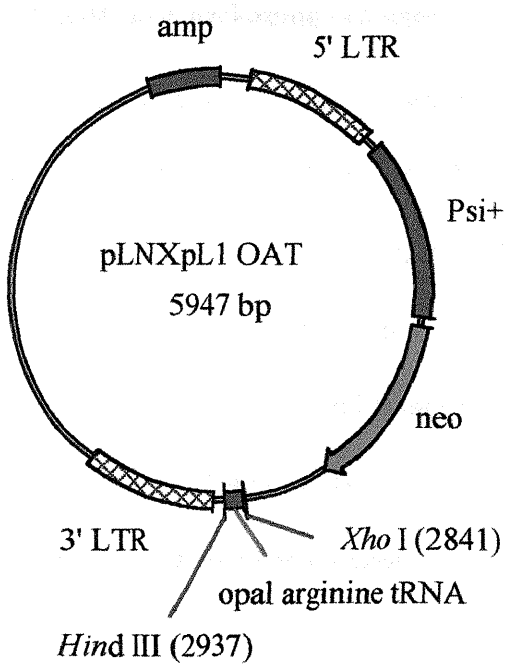

Figure 6. The synthetic opal arginine suppressor tRNA gene was subcloned into the pLNXpL1 retroviral vector to generate the recombinant plasmid pLNXpL1 OAT.

Large scale production of the recombinant pLNXpL1 OAT plasmid DNA

DH10B *E. coli* competent cells, transformed with recombinant plasmid constructs with functional suppressor tRNA, were incubated at 37°C overnight in a bacterial shaker in 500 ml LB-ampicillin (100 μg/ml) medium. Plasmid DNA was purified using a QIAGEN plasmid Maxi kit following the manufacturer's protocol. To ensure the quality of the maxiprep plasmid DNA, samples of the maxiprep DNA and the corresponding miniprep DNA were compared by electrophoretic patterns of restriction-digested DNA fragments, and verified by a functional activity assay for suppressor tRNA.

DNA sequencing of plasmid DNA constructs

DNA sequencing of recombinant plasmid DNA was performed by the DNA Sequencing Facility at Iowa State University, Ames, IA.

Transfection of GP+E-86 ecotropic packaging cell lines

GP+E-86 packaging cells at passage 7 were seeded into 6-well plates at 2 X 10^5 cells per 35 mm well. pLNXpL1 OAT7 and pLNXpL1 OAT8 were transfected into separate wells using 4 μg plasmid DNA and 12 μl Fugene 6 in the manner previously described. Transfection medium was aspirated from the GP+E-86, the cells were washed with 2 ml HBSS, and the medium replaced with 2 ml complete D-MEM. After 24 hr, the viral supernatant was used to transduce PA317 cells.

Transduction of PA317 amphotropic packaging cells

PA317 target packaging cells at passage 7 were seeded into separate 6-well plates 1 X 10^5 cells per 35 mm well, resulting in approximately 50% confluent cell growth. After

24 hr, the PA317 cells were transduced using the viral supernatant from GP+E-86, as previously described, for a total of six transductions. Twelve hr after the last transduction, the medium over the PA317 cells was removed, the cells were washed with 2 ml HBSS, and the medium replaced with 2 ml complete D-MEM plus G418 (1mg/ml). Cells were incubated in complete D-MEM plus G418 until no viable cells were detected in nontransduced PA317 cell wells (negative control), usually five to eight days.

Transduction of XP12ROSV human target cells

PA317 packaging cells producing recombinant pLNXpL1 OAT7 and pLNXpL1 OAT8, and XP12ROSV target cells were seeded into separate 6-well plates at 1 X 10^5 cells per 35 mm well, to generate a population of cells at 40-50% confluence in 24 hr. Transduction and selection of the XP12ROSV target cells were performed as described above for establishing the PA317 packaging cells.

Viral titers

Viral titers, a bioassay, were performed to determine the presence and indicate the quantity of infectious particles produced in the supernatant of the PA317 packaging cell lines transduced with plasmids containing the suppressor tRNA gene insert (Cepko et al., 1996). IGROV human ovarian carcinoma cells were seeded into 6-well plates at 2 X 10^5 cells per 35 mm well. PA317 vector producing cells were grown to 95% confluence in T80 culture flasks (Nunc). Medium was removed from the PA317 vector producing cells and replaced with 10 ml fresh complete D-MEM. After 24 hr, viral supernantant was removed from PA317 cells and centrifuged 10 min at 3000 X g to pellet any PA317 packaging cells. The supernatant was carefully removed to avoid pellet cells, and diluted

to 1/10, 1/100, 1/1000, and 1/10000 using complete D-MEM. Protamine sulfate, 10 μg/ml, was added to the serial dilutions of supernatant. Triplicate wells of IGROV cells were used for each serial dilution. Culture medium was removed from the IGROV cells and replaced with 1 ml diluted supernatant/complete D-MEM/protamine sulfate. IGROV cells were incubated at 37°C for 24 hr. The medium was aspirated from the IGROV cells. IGROV cells were washed with 2 ml HBSS and the medium replaced with 2 ml complete D-MEM plus G418 (1 mg/ml).

IGROV cell colonies were allowed to grow for seven days with one change of complete D-MEM plus G418 (1 mg/ml). The medium was aspirated from the IGROV cells. IGROV cells were washed twice with 2 ml HBSS. The IGROV cells were fixed and stained by adding 1 ml of 1% methylene blue (Sigma) dissolved in 100% methanol, and incubated for 30 min at RT. Excess stain was washed from the IGROV cells by inverting the plates under a gentle stream of deionized water, and the plates were allowed to dry.

Colonies of IGROV cells containing more than 40 cells were counted as a colony-forming unit (cfu). Dilution sets having between 20 and 120 colonies were counted. The IGROV colony counts were averaged for the triplicate wells at each dilution, then multiplied by the appropriate dilution factor to generate the report of cfu/ml of viral supernatant. Appropriate controls included nontransduced IGROV cells (negative control), and IGROV cells transduced with supernatant of known viral titer (positive control).

Multiplicity of Infection (MOI) is determined by dividing the number of cfu by the number of cells being transduced. This measurement can help to establish how much viral supernatant would be needed to transduce a quantity of cells under optimal conditions (Cepko, 1996).

RNA isolation

RNA was isolated using the single-step guanidinium acid-phenol method (Krieg, 1996; Chomczynski and Sacchi, 1987). All glassware and equipment were RNase decontaminated before starting by an additional wash in RNase Free (Continental Laboratory Products) and rinse with DEPC-treated water (0.2 ml DEPC to 100 ml double-deionized H_2O). In addition, all glassware and equipment was handled with gloved hands, and work was performed quickly (Gilman, 1994). All reagents were made using autoclaved, DEPC-treated, double-deionized H_2O to avoid RNase contamination.

Transduced cells were seeded into a 150 mm culture plate and grown to an estimated 1.8 X 10^7 cells. Medium was aspirated from cell cultures and the cells were lysed with 2 ml denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol). The lysate was passed seven times through an 18 gauge needle attached to a 5 ml syringe. 0.2 ml of 2 M Sodium acetate, pH 4, was added to the homogenate and vortexed 20 sec. 2 ml water-saturated phenol (100 g phenol crystals dissolved in 100 ml distilled H_2O at 60-65 ^oC) was added and the mixture vortexed 20 sec. Subsequently, 0.4 ml of 49:1 chloroform/isoamyl alcohol was added and the mixture vortexed 20 sec. The mixture was incubated 15 minutes over ice, then

centrifuged 20 min at 10,000 X g at 4°C. The aqueous phase was then transferred, by pipette, to a clean test tube. RNA was precipitated by adding 2 ml of 100% isopropanol. Samples were incubated 30 min at -20°C, centrifuged 10 min at 10,000 X g, 4°C, and the supernatant was discarded. The RNA pellet was dissolved in 0.6 ml denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol), precipitated with 0.6 ml of 100% isopropanol for 30 min at -20°C, centrifuged 10 min at 10,000 X g, 4°C, and the supernatant was discarded. The RNA pellet was resuspended in 0.6 ml 75% ethanol at RT, vortexed 20 sec, incubated 15 minutes at RT, and centrifuged five min at 10,000 X g, 4°C. The supernatant was discarded and the RNA pellet allowed to air dry. RNA was resuspended in 100 µl DEPC-treated water and quantitated by spectrophotometric measurement. 1 µl of resuspended DNA was added to 99 µl of TE, pipetted into a 100 µl cuvette and quantitated by spectrophotometer (Beckman DU Series 500) reading at OD_{260} using the manufacturer's protocol.

RNA slot blots

Samples were denatured using 10 µg total RNA and three volumes of denaturing mix consisting of 500 µl formamide, 162 µl 12.3M formaldehyde, and 100 µl MOPS buffer [0.2 M 3-(N-morpholino)-propanesulfonic acid, pH 7.0, 0.5 M sodium acetate, 0.01 M EDTA]; and incubated 15 min at 65°C. Two volumes of ice cold 20X SSC (175 g NaCl, 88.2 g sodium citrate, bring the volume to 1 liter with H_2O, pH 7) was then added to each RNA sample. The samples were kept on ice until they were loaded into the

manifold. A Nytran Plus membrane, pore size 0.45 μm (Schleicher & Schuel), and Whatman filter were cut to size, premoistened in DEPC-treated water, and soaked in 20X SSC for 30 min. The prepared Nytran Plus membrane and Whatman filter were then set into a SlotBlot model PR648 manifold (Hoefer Scientific Instruments) according to the manufacturer's directions. Each well was prefilled with 500 μl 10X SSC and drawn through at a rate of 10 min per ml. Prepared samples were then added to the wells and allowed to filter through the membrane. Empty wells were filled with 1 ml of 10X SSC. After samples wells were emptied, 200 μl 10X SSC was added twice and allowed to filter through the membrane. The vacuum was discontinued, the manifold disassembled, and the membrane carried between two Whatman filters for immediate crosslinking in the UV Stratalinker 1800 (Stratagene) using the autocrosslink setting. The membrane was soaked briefly in 200 ml 2X SSC then prehybridized eight hr or overnight at 42°C in 10 ml Hybrisol I (50% formamide, 10% dextran sulfate, and 1% SDS; Oncor, Inc.).

Opal Arg tRNA probe:

A probe for the opal arginine tRNA was constructed using the double-stranded suppressor tRNA gene insert as a template. A primer, BB6 5' cgagaaaacgaaccccacttaacc 3', consisting of the first 24 nucleotides of the antisense strand was used to generate a radiolabeled strand complementary to the opal arginine tRNA. The double-stranded DNA was denatured by heating 30 pmol DNA at 100°C for 10 min and immediately cooled on ice. A radiolabeled strand was synthesized in a reaction containing 150 pmol BB6 primer; dATP, dGTP, and dTTP 1:1:1 mixture, 0.05mM; 50 μCi [α^{32}P]dCTP (Amersham); and 4

units of Klenow enzyme and reagents accompanying the enzyme (Boehringer Mannheim); 0.5 M Tris HCl pH 7.5; 0.1 M $MgCl_2$; 10 mM DTT; 0.5 mg/ml BSA, adusted to a final volume of 25 µl with TE. The mixture was incubated 30 min at 37°C, and the reaction stopped by heating to 65°C for 10 min in a heat block. The probe was ethanol precipitated to remove unincorporated nucleotides and the pellet resuspended in 100 µl TE. 1 µl of probe was added to 4 ml of OptiScint 'HiSafe" scintillation fluid (LKB Scintillation Products) in a scintillation vial, and quantitated for ^{32}P activity in a Tri-carb 2100TR (Packard) scintillation counter using a direct reading of ^{32}P activity. The radiolabeled probe was added to 10 ml Hybrisol I (Oncor, Inc.) at a concentration of at least 1 X 10^6 cpm/ml of hybridization solution.

Hybridization of probe to RNA samples on Nytran membranes

Nytran membranes with total RNA samples were prehybridized with 10 ml Hybrisol I solution for eight hr or overnight at 42°C in a rolling hybridization cylinder (Hybridizer 700, Stratagene). The prehybridization mixture was removed and replaced with 10 ml of radiolabeled probe in Hybrisol I. Membranes were then hybridized eight hr or overnight at 42°C.

Membrane washes and autoradiography

Hybridized membranes were washed twice for five min using 250 ml 2X SSC and 0.1% SDS at RT on a shaking bath. A third 15 min wash using 250 ml 0.1X SSC and 0.1% SDS at 57°C in a shaking water bath was sufficient to remove any unbound probe. Membranes were briefly rinsed in 250 ml 2X SSC, wrapped in plastic film, and

autoradiographed for 24 hr at $-70^{o}C$ using a ^{32}P intensifying screen (Autoradiography Cassette FBXC 810, Fisher Biotech).

Restoration of functional activity of pLNCX mhRGFP gene

To demonstrate functional correction of the arg73opal mhRGFP gene, XP12ROSV cells expressing the mhRGFP gene were transduced with the suppressor tRNA gene. To introduce the mutated hRGFP first, 2 X 10^5 XP12ROSV cells were seeded into a 35 mm well. The cells were transfected overnight using 4 μg of mhRGFP DNA and 6 μl FuGENE 6. The transfection medium was aspirated, and the XP12ROSV cells were washed with HBSS. The XP12ROSV cells were incubated in 2 ml complete D-MEM for 24 hr and then selected with 2 ml complete D-MEM containing G-418 (100 mg/ml) until selection was complete in the negative control (nontransfected XP12ROSV cells) wells and XP12ROSV cells transfected with mhRGFP began to proliferate in complete D-MEM with G-418. The neomycin-selected population of XP12ROSV cells was then transduced with viral supernatant from PA317 cells containing pLNXpL1 OAT7 or pLNXpL1 OAT8.

The XP12ROSV cells, transfected with mhRGFP and transduced with either pLNXpL1 OAT7 or pLNXpL1 OAT8, were observed for GFP fluorescence. The medium was removed from the wells of the culture plate and replaced with 2 ml HBSS in each 35 mm well. The cultured cells were observed at 100 X magnification using a Diaphot 300 fluorescent microscope (Nikon) illuminated with a 488 nm light source. GFP-positive cells are easily visible as bright green cells.

RESULTS

Sequence verification

Sequence data revealed that two forms of opal arginine suppressor tRNA genes had been subcloned into pLNXpL1. pLNXpL1 OAT8, contained the synthetic suppressor tRNA gene inserted in the vector without any additional nucleotides or deletions. The second clone, pLNXpL1 OAT7 contained a tandem repeat of the gene insert without any additional nucleotides between the pair of suppressor tRNA genes. Both inserts were oriented in the vector in the same direction, 5' *Xho* I to 3' *Hin*d III. Both recombinant plasmid clones were used in this study.

Production of infectious particles

Virion production by packaging cells is measured by means of a viral titer bioassay. The results allow one to determine the amount of virus used in experiments and also identify cell lines with high viral titer. The viral titer is reported as colony forming units per ml of supernatant (cfu/ml), a measure based on the production of a selectable colony of cells. The viral titer is considered a rough quantitative means where titers within a factor of 3 are considered similar due to variation in the number of cell divisions which may occur after viral integration and before the addition of selective media. If greater accuracy is needed, the viral titer bioassay should be performed multiple times and the results averaged (Cepco, 1996). In this study, viral titer was used to indicate the presence of infectious particles containing the neomycin-resistance gene subcloned with the suppressor tRNA gene. Viral titers would indicate that transfer and expression of the opal

arginine tRNA would not adversly affect the production of virions by readthrough of normal stop codons.

Multiplicity of Infection (MOI), determined by dividing the number of cfu by the number of cells being transduced, can help establish how much viral supernatant would be needed to transduce a quantity of cells. A mixed population (cells transduced but not cloned) of PA317 packaging cells producing virions from pLNXpL1 OAT8 were reported to have a viral titer average of 5.33 X 10^4 cfu/ml of viral supernatant, and pLNXpL1 OAT7 were reported to have a viral titer average of 1.8 X 10^3 cfu/ml of viral supernatant. The XP12ROSV human target cells, were plated at 1 X 10^5 cells per 35 mm well and transduced with supernatant six times over three days. Transductions using PA317 producing virions from pLNXpL1 OAT8 would have been over one MOI (5.3 X 10^4 cfu/ 1 X 10^5 cells = 1.59 MOI). Transductions using PA317 producing virions from pLNXpL1 OAT7 would have had considerably less than one MOI (5.4 X 10^3 cfu/ 1 X 10^5 cells = 0.054 MOI).

Expression of the suppressor tRNA

Slot blots. To determine the expresson of suppressor tRNA in PA317 packaging cells and XP12ROSV human target cells, the slot blot was probed with ^{32}P-labeled oligonucleotide. Expression of the suppressor tRNA was observed in both PA317 (Figure 7) and XP12ROSV target cells (Figure 8).

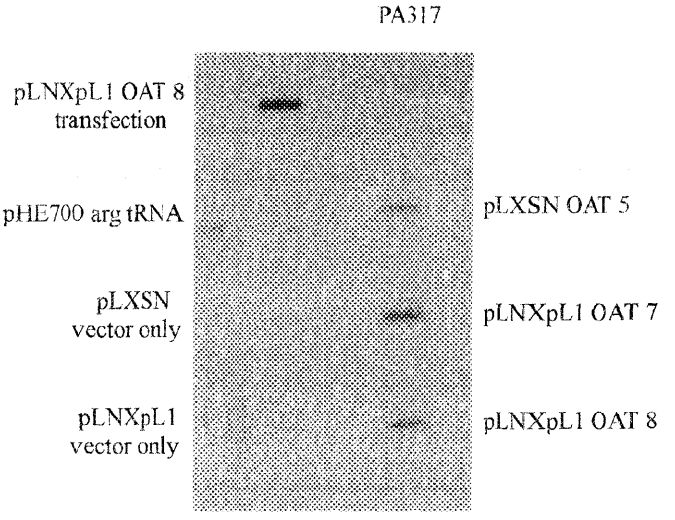

Figure 7. Slot blot showing opal arginine tRNA detected by the radiolabeled probe in total RNA samples from transduced PA317 packaging cells. The left column contains control RNA samples. pLNXpL1 OAT8 is the positive control obtained from PA317 cells transfected with the pLNXpL1 OAT8 plasmid. pHE700 arg tRNA is a negative control for probe specificity and demonstrates that the probe used in this study was sensitive to a single base pair change corresponding to the nucleotide change in the anticodon of arg tRNA (UCG) to the anticodon of opal arg tRNA (UCA) and specific for the opal arg suppressor tRNA. pLXSN and pLNXpL1 are negative controls for plasmids without opal arg tRNA gene inserts. The right column contains study samples of RNA from PA317 cells transduced by GP+E-86 packaging cells producing virions from recombinant plasmids with opal arg tRNA gene inserts. pLXSN OAT5 is a recombinant plasmid containing an opal arg tRNA gene insert identical to pLNXpL1 OAT8 and shows similar expression of the gene without regard to plasmid vector. pLNXpL1 OAT7 contains a complete tandem repeat of the opal arg tRNA gene insert. pLNXpL1 OAT8 contains a single copy of the opal arg tRNA gene insert. pLXSN OAT5, pLNXpL1 OAT7 and OAT8 all demonstrated expression of the opal arg tRNA gene insert.

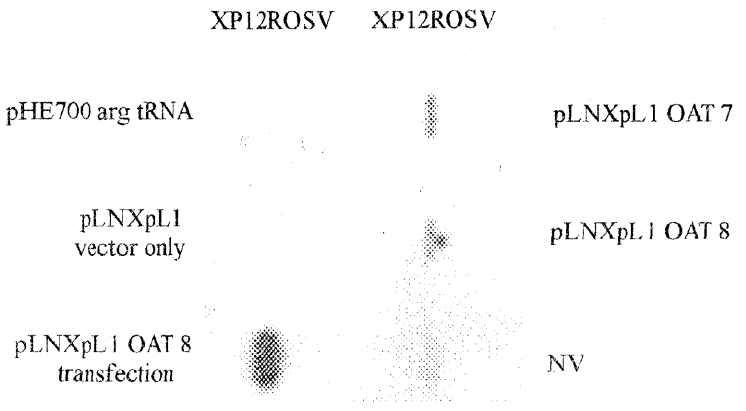

Figure 8. Slot blot showing opal arginine tRNA detected by the radiolabeled probe in total RNA samples from XP12ROSV human target cells transduced from PA317 packaging cells. The left column contains total RNA from control samples. pHE700 arg tRNA is a negative control for probe specificity. No probe signal was detected from the RNA of XP12ROSV cells transfected with a vector including the gene for arg tRNA. pLNXpL1 is a negative control for cells transduced from PA317 producing virions from plasmids without the opal arg tRNA gene insert. pLNXpL1 OAT8 transfection is a positive control for opal arg tRNA expression. The top two samples of the right column contains total RNA samples from XP12ROSV cells transduced by PA317 producing virions from recombinant plasmids with the opal arg tRNA gene insert. Opal arg tRNA expression is evident in XP12ROSV cells transduced with virons from both pLNXpL1 OAT7 and pLNXpL1 OAT8. NV, a negative control, is a total RNA sample from XP12ROSV cells that have not been transfected or transduced.

Results indicate GP+E-86 and PA317 vector producer cells were able to produce virions from the recombinant plasmids pLNXpL1 OAT7 and pLNXpL1 OAT8 containing the opal arg tRNA gene insert. GP+E-86 and PA317 vector producer cells successfully delivered the suppressor tRNA gene to human target cells. XP12ROSV cells transduced by virions from the recombinant plasmids pLNXpL1 OAT7 and pLNXpL1 OAT8 translated the opal arg tRNA gene insert as a functional suppressor tRNA. Expression of

the opal arg suppressor tRNA was not detected in PA317, XP12ROSV, and XP12ROSV cells or these cell lines transiently transfected with pLNXpL1 vector which did not contain the opal arg tRNA gene insert. PA317 and XP12ROSV cells transfected with arginine tRNA and selected with hygromycin (a negative control to demonstrate the specificity of the radiolabeled probe) did not show any probe signal on RNA slot blots. An intense signal was observed in PA317 and XP12ROSV cells transiently transfected with pLNXpL1 OAT8 (a positive control for opal arg tRNA gene expression).

Translation of a functional protein from a gene with a nonsense mutation

To demonstrate successful delivery, expression and suppression of the nonsense mutation by the suppressor tRNA; XP12ROSV human target cells were transfected with the pLNCX arg73opal mhRGFP gene, selected with neomycin, and transduced with the retroviral supernatant containing the suppressor tRNA gene (Figure 9).

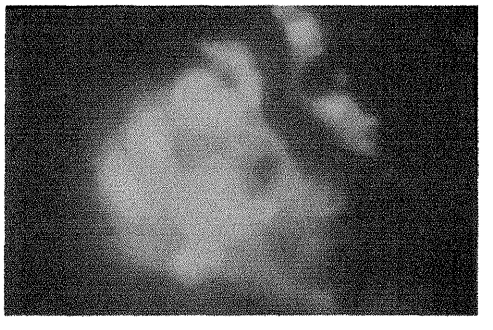

Figure 9. XP12ROSV human target cells transfected with the mhRGFP gene, selected with neomycin, and transduced with pLNXpL1 OAT demonstrate translation of functional GFP protein.

Restoration of GFP fluorescence was observed to range from approximately 1% of the cells to only a few isolated cells in a 35 mm well. No functional correction of mhRGFP was ever observed in cells transfected with mhRGFP only, or transduced with

pLNXpL1 OAT only.

DISCUSSION

The present study demonstrates that recombinant retroviral vectors may have the potential to deliver the suppressor tRNA genes to human target cells using currently accepted methods. Expression of the suppressor tRNA gene was observed in both the packaging cell lines and human target cells. Thus, demonstrating that opal arg suppressor tRNA gene could be packaged into retroviral virions, delivered to the target cells, and subsequently expressed with the ability to suppress the nonsense mutation in the mhRGFP gene. However, the functional correction of the mhRGFP was observed in only 1% of the transduced XP12ROSV cells and would need to be improved for therapeutic effect.

The low percentage of functional correction could have been due to a low mhRGFP mRNA copy number which has not been studied in this investigative model. Low mRNA copy number has been associated with nonsense mutations in many organisms, however, the role of translation in nonsense-mediated mRNA decrease has not been determined in mammalian cells (Buzina and Shulman, 1999). In addition, eukaryotic release factor 1 has been shown to compete with suppressor tRNAs at termination codons, and thereby generate truncated mRNA which would be translated to truncated, nonfunctional protein molecules (Drugeon et al., 1997).

There are several ways the system investigated in this study could possibly be improved. The synthetic suppressor tRNA gene could be redesigned to increase the level of expresison by linking multiple copies of the suppressor tRNA gene in tandem. The most appropriate retroviral vector to carry the mhRGFP gene to a specific tissue could be

determined. The best vector producer cells could be isolated. Or only those mRNA sequences with nonsense mutations in a suppression-favorable codon context could be studied for therapeutic efficacy.

Review design of suppressor tRNA gene. The suppressor tRNA gene could be redesigned to optimize the expression of the gene and function of the tRNA transcript. The synthetic suppressor tRNA gene in this study consisted of the structural sequence and 15 of the 3' base pairs. The arginine tRNA gene should be studied to determine which specific regions contribute to optimal expression of the gene and those structural features essential for aminoacyl-tRNA synthetase recognition.

The 5' and 3' flanking sequences have been reported to have a significant effect in gene transcription. Arnold and Gross (1987) reported that the 5' extragenic control region between positions of -51 and -16 acted as a positive modulator for transcription factor binding when expressing human valine tRNA genes. In another study, Tapping et al. (1994), detailed how transcription was partially inhibited or initiation redirected if the -43 and -46 positions of the human serine tRNA gene were prebound with *lac* repressor. They determined that the human RNA polymerase III transcription complex extends at least 35 nucleotides upstream to the structural coding region and that this flanking region was important in the assembly of the initiation complex. The 3' flanking sequence of the human valine tRNA gene has a role in stabilizing preinitiation complex formation. Arnold et al. (1988), described distinct roles for the 3' and 5' flanking sequences in a two-step mechanism where transcription factor TFIIIC associated with the tRNA and an extragenic control region-independent conformational change occurred.

The function of the tRNA could be optimized by assuring the presence of the recognition features needed for aminoacyl synthetase interaction. Synthetases, which attach amino acids to tRNA, appear to be recognizing elements in the anticodon region, the acceptor stem, and the "discriminator base" at position 73 (Saks et al., 1994). Current sequences of the arginine tRNA gene and synthetase structures should be reviewed and compared to the synthetic tRNA gene.

Introduce more copies of the suppressor tRNA gene. The low level of functional correction in the transduced cells relative to the cotransfected cells could have been the result of only a single copy of the tRNA gene integrated into the target cell genome. Multiple copies of the suppressor tRNA gene could be subcloned into the retroviral vector and thereby improve the level of expression by increasing the number of copies available for transcription. Studies should be done to determine the optimal length of the linker regions between genes.

Selection of the appropriate vector. Vectors are continuously being developed to include new markers, a variety of promoters for special needs, conditional expression, and species-specific elements. Only one type of retroviral vector system was used in this study, so it is possible that another retroviral vector or perhaps an entirely different vector system, such as one based on the adenovirus or lentivirus, may be more suitable for suppressor tRNA gene transfer. A retroviral vector was selected for this study in order to stably integrate the suppressor tRNA gene into the host DNA, however, it also requires mitotic cell division for transduction (Robbins and Ghivizzani, 1998). Some current applications for retroviral vectors target rapidly dividing cells such as hepatocytes,

proliferating synovial cells, tumor cells, or *ex vivo* transductions of cells which can be later transplanted into a recipient. Therefore, selection of a vector should consider stable gene expression and the nature of the target tissue.

The vector efficiency may also be somewhat impaired by expression of a suppressor tRNA gene. At least one investigator has reported difficulty in establishing a vector system which would carry an opal suppressor tRNA. Capone et al. (1985), reported an inability to establish SV40 virus stocks carrying an opal serine suppressor tRNA. Their examination of the SV40 DNA sequence indicated that readthrough of a UGA stop codon in VP1, a major capsid protein, would produce a protein at least 40 amino acids longer. They speculated that the additional protein length interfered with viral assembly. Production of infectious particles is essential for gene transfer using retroviral vectors. Adequate viral titers and the presence of opal arginine suppressor tRNA in target cells would provide evidence that infectious particles were produced and that this retroviral vector system had the potential to transfer an opal suppressor tRNA. However, it may be beneficial to explore alternative vectors for optimal transfer.

Isolation of a high-titer vector-producing clone. The transductions in this study were made from a mixed population (cells transduced but not cloned) of vector-producing cells. Retroviral vectors will randomly incorporate the gene of interest into the host cells' genome. It is entirely possible that integration into a key regulatory gene would be lethal or debilitating for both packaging cells or target cells. Packaging cells should be cloned by limiting dilution in order to isolate an optimal vector producer cell clone.

Selection of appropriate mRNA transcripts. Some mRNA codon contexts will inhibit suppression of nonsense codons. Miller and Albertini (1983) studied the efficiency of amber suppression in 14 different UGA codons and noted correlation with the first base on the 3' side. They determined, with some exceptions, that nonsense codons followed by G or A were suppressed more efficiently than nonsense codons followed by C or U. In addition, secondary and tertiary structure of mRNA surrounding the nonsense codon may affect the suppression efficiency. In a study examining infectivity and replication properties of MoMuLV, a single base change in the sequence flanking the amber codon at the *gag-pol* junction resulted in a change in the secondary structure and thereby affecting the translational efficiency. It is also noted that because of this change, a MoMuLV-based vector may not be suitable for the transfer of amber suppressor tRNA.

Suppressor tRNA genes as therapeutic agents. The proposed use of suppressor tRNA genes as therapeutic agents may have two advantages over a strategy to introduce a normal gene. First, a tRNA gene is relatively small and well within the carrying capacity of retroviral vector systems. Many genes are much larger than the typical size of the retroviral genome. Genes transferred by retroviral vectors would be limited to approximately 11 kb. Second, natural tRNA genes are expressed in all cells because they are essential to protein synthesis while protein genes may not be expressed in all cell types.

CONCLUSION

Recombinant retroviral vectors, assisted with packaging cell lines, were able to transfer opal arginine suppressor tRNA genes into human target cells. The transferred genes were expressed as suppressor tRNA with the ability to mediate translation of a functional protein from a hRGFP gene with a nonsense mutation. However, functional correction of mhRGFP was minimal and will require further study in order to develop a system with therapeutic benefit.

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PRESENTATION OF RESEARCH

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